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Identification and characterization of the novel protein CCDC106 that interacts with p53 and promotes its degradation

Jianlin Zhou [1], Xi Qiao [1], Ling Xiao, Wei Sun, Lin Wang, Hong Li, Yuan Wu, Xiaofeng Ding, Xiang Hu, Chang Zhou, Jian Zhang *

Key Laboratory of Protein Chemistry and Developmental Biology of Education Ministry of China, College of Life Science, Hunan Normal University, Changsha, Hunan 410081, China

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The putative CCDC106 protein was previously identified as a p53-interacting partner by automated yeast two-hybrid screening, but its sequence and function have not been validated experimentally. Here, we identified three variant transcripts of the CCDC106 gene; these transcripts differ in their second exons due to the use of different splice acceptor site, but encode an identical protein of 280 residues. A bipartite nuclear localisation signal at residues 151–164 mediates the nuclear localisation of CCDC106. Double immunofluorescence staining revealed the colocalisation of endogenous CCDC106 and p53 protein in nuclei. The in vivo interaction between CCDC106 and p53 was confirmed by a co-immunoprecipitation assay. Furthermore, we demonstrated that CCDC106 promotes the degradation of p53 protein and inhibits its transactivity.

Structured summary:
MINT-7681390: *CCDC106* (uniprotkb:Q9BWC9) *physically interacts* (MI:0915) with *p53* (uniprotkb:P04637) by *anti tag co-immunoprecipitation* (MI:0007)
MINT-7681212: *p53* (uniprotkb:P04637) and *CCDC106* (uniprotkb:Q9BWC9) *colocalise* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

The tumour suppressor protein p53 maintains genomic integrity and cellular homeostasis. In response to stress signals such as DNA damage, oncogenic stimuli or hypoxia, p53 accumulates in the cell and becomes activated. It then initiates an anti-proliferation and pro-apoptotic program that prevents the multiplication of damaged and potentially pre-cancerous cells [1,2]. Lack of p53 function is often connected with development of cancer [3]. Inactivation of p53 by mutation occurs in over 50% of all human cancers [4,5]. In cancer types that harbour wild-type p53, p53 function is frequently abolished by overexpression of negative regulators such as MDM2 (mouse double minute 2) [6,7]. MDM2 is an important regulator of p53. It catalyses p53 ubiquitination, marking p53 for degradation by the proteasome [8,9]. Apart from MDM2, p53 can be ubiquitinated by several other ubiquitin ligases, such as COP1 (constitutively photomorphogenic 1), PirH2 (p53-induced RING-H2 protein) and synoviolin [10–13]. The stability of p53 is also controlled by physical interactions with other proteins. Although these interacting proteins cannot modify p53 themselves, they can impinge on p53 modifications by enhancing or reducing the association of p53 with modifying enzymes [13]. For example, some p53-interacting partners such as ING1b and activating transcription factor 3 (ATF3) disrupt the interaction between MDM2 and p53 and thus reduce p53 ubiquitination [13–15]. Other interacting partners, such as Yin Yang 1 (YY1) and focal adhesion kinase (FAK), increase p53 ubiquitination and degradation by stabilizing the interaction [13,16,17]. These results suggest that identification of novel interacting partners of p53 will provide further insight into the regulation of p53 activity.

The putative coiled coil domain containing 106 protein (CCDC106, previously termed HSU79303 [18]) was identified as a p53-interacting partner by automated yeast two-hybrid screening [18], but its sequence and interaction with p53 have not been further confirmed. Furthermore, the cellular function generated by the interaction between CCDC106 and p53 is unknown. In this paper, we identified three variant transcripts of the CCDC106 gene in HeLa cells and a bipartite nuclear localisation

* Corresponding author. Fax: +86 731 88872792.
E-mail address: zhangjian@hunnu.edu.cn (J. Zhang).

[1] These authors contributed equally to this work.

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signal (NLS) within the CCDC106 protein, and demonstrated that CCDC106 interacts with p53 in vivo and promotes the degradation of the p53 protein.

2. Materials and methods

2.1. Plasmids construction

The coding sequence of human CCDC106 was amplified with primers ORF-EcoRI-F and ORF-KpnI-R (Table 1) and cloned into pCMV-Myc and pEGFP-C3 at *Eco*RI and *Kpn*I sites, resulting in CCDC106 fusion protein expression plasmids pMyc-CCDC106 and pEGFP-CCDC106. For generating the NLS (residues 151–164) deletion mutant pEGFP-ΔNLS, the N-terminus (residues 1–150) and C-terminus (residues 165–280) of CCDC106 were amplified from pEGFP-CCDC106 (primers for N-terminal PCR: ORF-EcoRI-F and NLSdel-R, primers for C-terminal PCR: NLSdel-F and ORF-KpnI-R; NLSdel-F and NLSdel-R contain complementary overhangs), followed by annealing and second amplification with primers ORF-EcoRI-F and ORF-KpnI-R. The final PCR products were then cloned into the *Eco*RI and *Kpn*I sites of pEGFP-C3. The site-specific NLS mutant plasmids pEGFP-mutNLS1(^{151}RRR → ^{151}AAA), pEGFP-mutNLS2(^{162}RRR → ^{162}AAA) and pEGRP-mutNLS(^{151}RRR…^{162}RRR → ^{151}AAA…^{162}AAA) were generated by overlapping extension PCR as previously described [19]. The coding sequence of p53 was cloned into pCMV-HA to generate the HA-tagged p53 expression plasmid pHA-p53. The luciferase reporter plasmid pp53-TA-Luc containing the p53 response element was purchased from BD Biosciences Clontech (Palo Alto, CA, USA).

2.2. Cloning the full-length cDNA of CCDC106 by RLM-RACE

The putative coding sequence of CCDC106 was amplified from HeLa first-strand cDNA using primers ORF-F and ORF-R (Table 1), and the amplified fragment was cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced. Based on the coding sequence, gene-specific primers were designed to clone the 5′-end and 3′-end sequences using the Firstchoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. Briefly, first-strand RACE-ready cDNA was synthesized with total RNA extracted from HeLa cells. Subsequently, RACE-PCR and nested RACE-PCR were performed with gene-specific primers (Table 1) and universal primers provided in the kit. The final PCR products were cloned into the pMD18-T vector and sequenced.

2.3. Cell culture, transfection and luciferase assay

HeLa cells were grown in a 24-well plate to 80% confluence and transfected with 0.2 μg of pp53-luc, 0.2 μg of pCMV-β (Clontech) and increasing amounts of pMyc-CCDC106 (0, 0.1, 0.2, 0.4 μg) using Lipofectamine 2000 (Invitrogen). At 36 h after transfection, the cells were lysed to measure luciferase activity using the luciferase assay system (Promega) as previously described [19]. Luciferase activities were normalized according to β-galactosidase activities.

2.4. Antibodies

Rabbit polyclonal antibodies to human CCDC106 were raised using a peptide (^{2}NDRSSRRRTMKDD^{14}C) conjugated to Keyhole Limpet Hemocyanin (KLH) and purified by affinity purification by Signalway Antibody Co., Ltd. (Nanjing, China). Rabbit anti-Myc polyclonal antibody, mouse anti-HA monoclonal antibody, mouse anti-p53 (Pab 1801) monoclonal antibody, and all the secondary antibodies for Western blotting or immunofluoresence staining were purchased from Santa Cruz.

2.5. Subcellular localisation analysis by fluorescence microscopy

HeLa cells were seeded on glass coverslips and treated as described previously [20]. To detect the localisation of expressed EGFP-fusion proteins, HeLa cells were transfected with individual EGFP-fusion protein expression plasmids. At 24 h post-transfection, the cells were fixed, and fluorescent signals were revealed under the fluorescence microscope (Axioskop 2, Carl Zeiss). To detect the localisation of endogenous p53 and CCDC106 protein, cells were processed and sequentially incubated with primary antibody and fluorescently labelled secondary antibody as described previously [20]. The murine anti-p53 monoclonal antibody and Texas Red-conjugated anti-mouse IgG (red) were used to detect p53, while rabbit anti-CCDC106 polyclonal antibody and FITC-conjugated anti-rabbit IgG (green) were used to detect CCDC106. Nuclei were stained by Hoechst 33258 (blue).

2.6. siRNA treatment

Three siRNAs designed against different regions of the human CCDC106 gene were synthesized (#1 forward 5′-GUCGGAGGCGGACAAUGAATT-3′ and reverse 5′-UUCAUUGUCCGCCUCCGACTG-3′, #2 forward 5′-GGGAGAGGCAGCGAGUGAATT-3′ and reverse 5′-UUCACUCGCUGCCUCUCCCGG-3′, #3 forward 5′-CGCGUGAGACA-

Table 1
Primers used in this study.

Name	Sequence (5′–3′)	Purpose
ORF-EcoRI-F[a]	GAATTCCCATGAATGACCGGAGCA	ORF cloning
ORF-KpnI-R	GGTACCGTGCGATCACCGCTTGAA	
NLSdel-F	GCCAGTGAGTTTGGGAAGCCCAAGGCCCG	Deletion mutagenesis of NLS
NLSdel-R	CTTCCCAAACTCACTGGCGCTGCCTTCTTC	
NLSmut1-F	AGTGAGGCGGCGGCGCAGAAG	Point mutagenesis of NLS1
NLSmut1-R	CTTCTGCGCCGCCGCCTCACT	
NLSmut2-F	AGTGCGGCGGCCTTTGGGAA	Point mutagenesis of NLS2
NLSmut2-R	TTCCCAAAGGCCGCCGCACT	
ORF-F	CCATGAATGACCGGAGCA	ORF amplification
ORF-R	GTGCGATCACCGCTTGAA	
5′ CCouter	AGGCTCCTCGAAGTTTCTCC	5′-RACE
5′ CCinner	CTCATCGTCCTTCATTGTCC	5′-RACE nested PCR
3′ CCouter	ACTCCCGCCGCTGCTTTCTG	3′-RACE
3′ CCinner	GAGCAAGCTGCTGCTGCCCA	3′-RACE nested PCR
Exon1-F	GTCCCCGAGCCGGATTCC	Amplification of variants
Exon4-R	GGCGGAGGACGCAGCCT	

[a] F indicates forward primer, R indicates reverse primer.

GAGAAUUATT-3′ and reverse 5′-UAAUUCUCUGUCUCACGCGTT-3′). Non-sense siRNA (forward 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse 5′-ACGUGACACGUUCGGAGAATT-3′) was used as negative control. HeLa cells were transfected with siRNA at a final concentration of 100 nmol/L using Lipofectamine 2000 (Invitrogen). At 36 h after transfection, cell lysates were extracted for Western blotting to assess relative CCDC106 protein level.

2.7. Co-immunoprecipitation (co-IP) and Western blotting

Co-IP and Western blotting were performed as previously described [20]. Briefly, HeLa cells were transfected with pMyc-CCDC106 and pHA-p53 plasmids. At 24 h after transfection, cell lysates were prepared and immunoprecipitated with rabbit anti-Myc polyclonal antibody or preimmune rabbit IgG, and the precipitated protein was detected by Western blot analysis using the anti-HA monoclonal antibody.

2.8. Analysis of the influence of CCDC106 on level and degradation of p53 protein

To examine the influence of CCDC106 on the level of p53 protein, HeLa cells grown on a 6 cm plate were co-transfected with 2 μg of pHA-p53 and increasing amounts of pMyc-CCDC106 (0, 2, 4, 8 μg) and harvested at 8 h post-transfection. To detect the influence of CCDC106 on the degradation of p53 protein, 2 μg of pHA-p53 and 4 μg of pMyc-CCDC106 or empty vector pCMV-Myc were transfected into HeLa cells. At 8 h post-transfection, transfected cells were incubated with either cycloheximide (CHX, 50 μg/ml) for different lengths of time (0, 1, 2 and 4 h), or CHX plus increasing concentrations of MG132 (0, 10, 20, 40 μM) for 1.5 h. Cell lysates were prepared and separated by 10% SDS–PAGE electrophoresis. PageRuler™ prestained protein ladder (Fermentas) was used as molecular weight marker. A single blot membrane was cut into strips based on molecular weight and incubated with anti-HA, anti-Myc and anti-Actin antibodies to detect the protein levels of HA-p53, Myc-CCDC106 and β-Actin, respectively.

3. Results and discussion

3.1. Identification of three variant transcripts of the CCDC106 gene

The sequence of the CCDC106 gene is available in GenBank (accession no. NM_013301), but this sequence has not been confirmed experimentally. To validate the sequence of the CCDC106 gene, we amplified via PCR and sequenced the putative coding sequence of CCDC106. Based on the confirmed coding sequence, gene-specific primers were then designed and used to extend the 5′- and 3′-flanking sequences by RLM-RACE. We identified three fragments in the 5′-RACE reaction and one fragment in the 3′-RACE reaction. After sequencing, the three fragments of 5′-flanking sequences, the 3′-flanking sequence and the coding sequence were assembled into three different full-length cDNAs, representing three variant transcripts. The sequence data of these three transcripts were submitted to GenBank with the accession numbers GU354321, GU354322, GU354323, respectively. BLAST searching of the human genomic database using the sequences of the three variant transcripts showed that these three variant transcripts are transcribed from the same transcript start site, and each consists of six exons, but these transcripts differ in their second exons due to the use of an alternative splice acceptor site of exon 2 (Fig. 1B). To further confirm the in vivo expression of these three variant transcripts, we designed primers able to amplify the three transcripts and performed RT-PCR using HeLa cDNA as template. As shown in Fig. 1A, the amplification produced three different products: 232 bp, 288 bp and 476 bp, corresponding to variant transcript 1, 2, and 3, respectively. All these amplicons were further confirmed by sequencing.

We next examined the open reading frame (ORF) using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and found that

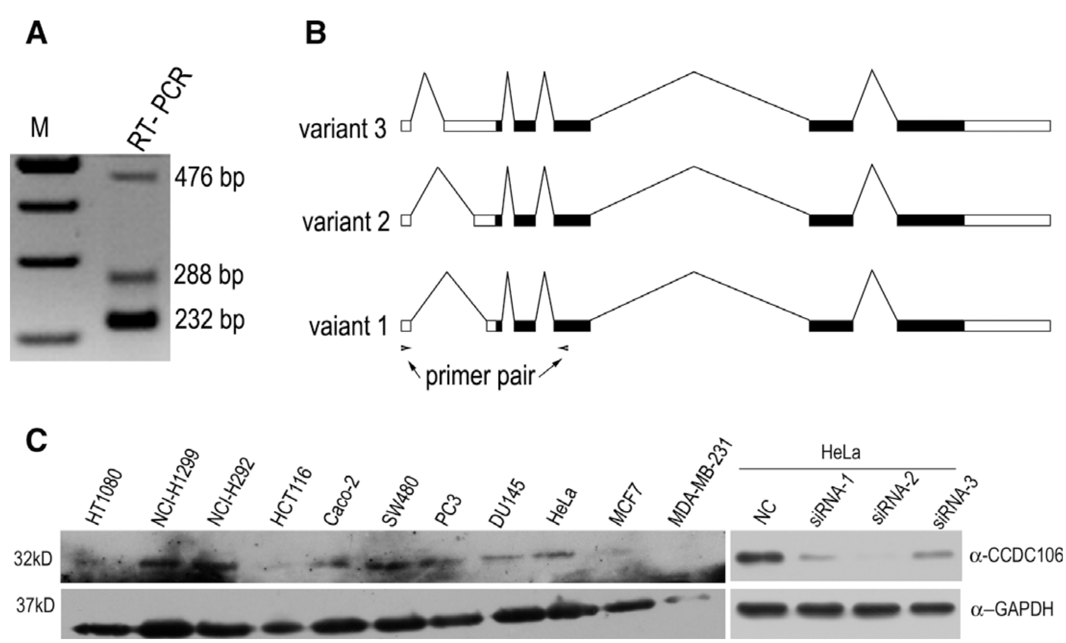

Fig. 1. In vivo identification of variant transcripts and protein of CCDC106. (A) RT-PCR analysis of HeLa RNA that identifies the transcript 1 (232 bp), 2 (288 bp) and 3 (476 bp). (B) Schematic representation of variant transcripts. The exons and introns are shown as boxes (open boxes for untranslated regions, black boxes for coding regions) and lines, and the RT primer positions are indicated by arrows. (C) Endogenous CCDC106 protein was determined by Western blotting with rabbit polyclonal anti-CCDC106 antibody in several cell lines and HeLa cells transfected with non-sense siRNA (NC) or CCDC106 siRNA (siRNA-1, siRNA-2, siRNA-3). The same blot membrane was then stripped and rehybridised with anti-GAPDH antibody as loading control.

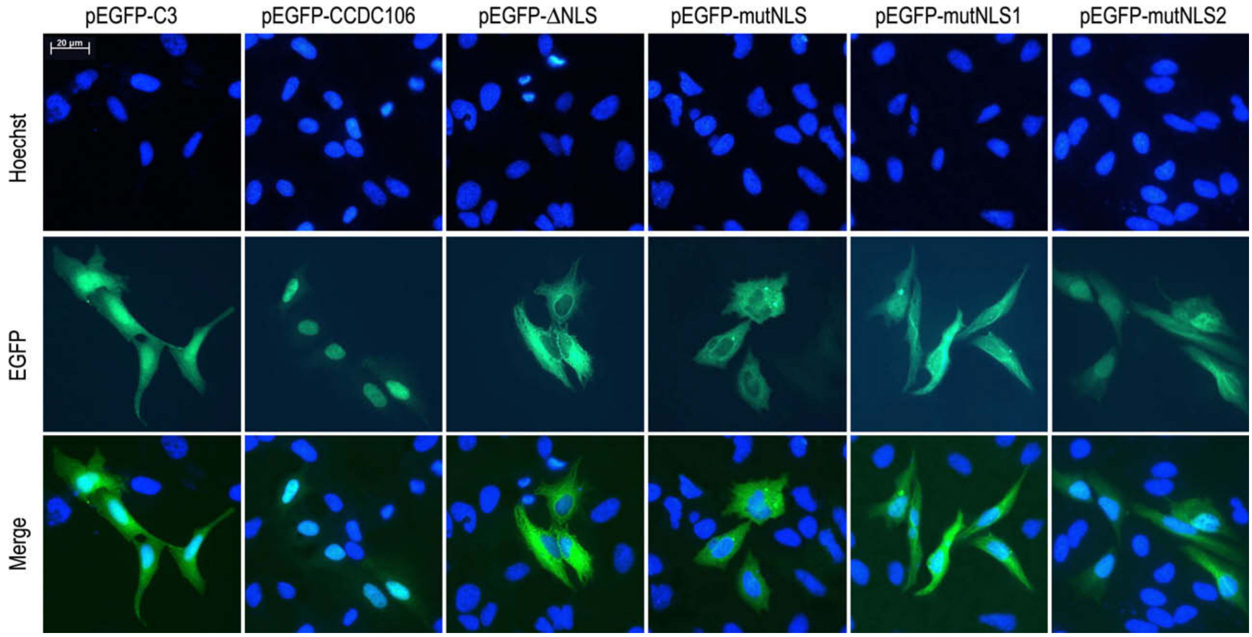

Fig. 2. Mutation of the NLSs abolishes the nuclear localisation of CCDC106. The EGFP-fusion protein expressing plasmids and empty vector pEGFP-C3 were transfected into HeLa cells, and EGFP fluorescence was examined directly 24 h post-transfection by fluorescence microscopy. Nuclei were stained by Hoechst 33 258. Merge represents the combined image of EGFP fluorescence and nucleus staining.

these three variant transcripts have an identical ORF of 840 bp, which encodes a protein of 280 amino acids with a calculated molecular weight of 32 kDa. To confirm this prediction, the rabbit CCDC106 polyclonal antibody was used for Western blotting to examine endogenous CCDC106 protein in cell lines. As shown in Fig. 1C, a band of ~32 kDa was detected in lysates of cells, consistent with the predicted size. From the relative intensities of bands for CCDC106 and GAPDH, we found that CCDC106 was predominately expressed in cell lines NCI-H 1299, NCI-H292 and SW480, while reduced expression of CCDC106 was also detected in cell lines Caco-2, PC3, Du145 and HeLa. To validate the specificity of the CCDC106 polyclonal antibody raised by us, three siRNAs against different regions of the human CCDC106 gene were synthesized and transfected individually into HeLa cells. As shown in Fig. 1C, all these CCDC106 siRNAs can effectively inhibit CCDC106 expression as determined by the rabbit CCDC106 polyclonal antibody, suggesting that the antibody is specific to CCDC106 protein.

3.2. A bipartite NLS mediates the nuclear localisation of CCDC106

To determine the subcellular localisation of the CCDC106 protein, we transiently transfected the EGFP-CCDC106 fusion protein expression plasmid or EGFP empty vector into HeLa cells. The localisation of the EGFP or EGFP-fusion protein in living cells was detected by fluorescence microscopy at 24 h post-transfection. As shown in Fig. 2, EGFP protein was observed in both the cytoplasm

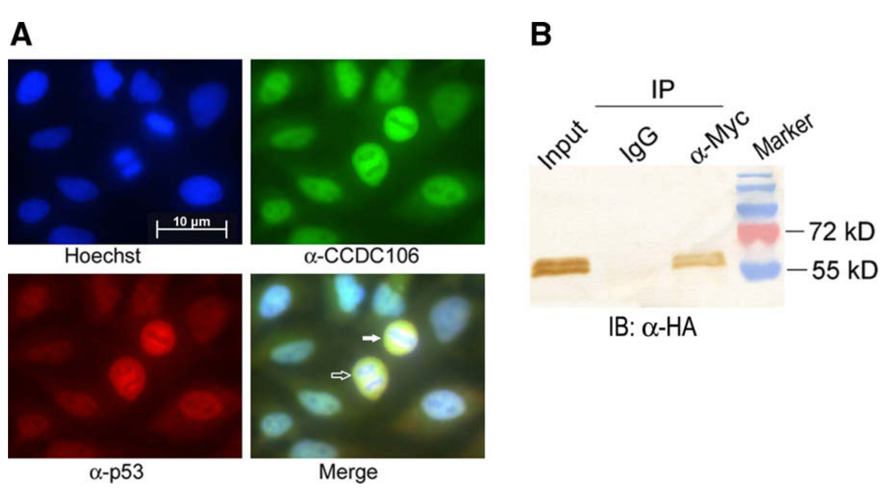

Fig. 3. CCDC106 co-localises with and interacts with p53. (A) Colocalisation of endogenous CCDC106 protein and p53 protein in HeLa cells. The murine anti-p53 monoclonal antibody and Texas Red-conjugated anti-mouse IgG (red) were used to detect p53, whereas rabbit anti-CCDC106 polyclonal antibody and FITC-conjugated anti-rabbit IgG (green) were used to detect CCDC106. Nuclei were stained by Hoechst 33258 (blue). Yellow in merged image (indicated by arrows) represents colocalisation of p53 and CCDC106 (white and open arrows show the localisation during metaphase and anaphase of cell cycle, respectively). (B) Detection of protein interaction between CCDC106 and p53 by co-IP assay. HeLa cells were transfected with pMyc-CCDC106 and pHA-p53 plasmids. At 8 h after transfection, cell extracts (800 µg) were prepared and immunoprecipitated (IP) with rabbit anti-Myc polyclonal antibody or control preimmune rabbit IgG. Coprecipitating HA-p53 was detected by immunoblotting (IB) analysis with the anti-HA monoclonal antibody. A total of 50 µg of cell extract was used as positive control (Input). Marker, prestained protein molecular weight marker.

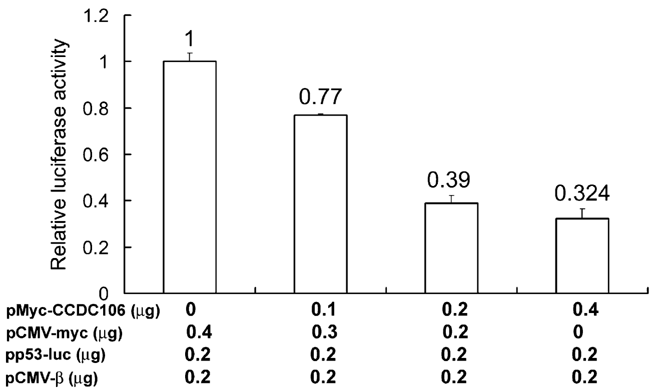

Fig. 4. Inhibitory effect of CCDC106 expression on p53 transcription activity. HeLa cells were co-transfected with pp53-luc and increasing amounts of pMyc-CCDC106 as indicated. Luciferase activities were measured 36 h post-transfection. Data (means ± S.D.) were represented as the fraction of activity relative to that observed in cells without pMyc-CCDC106.

and the nucleus, but EGFP-CCDC106 fusion protein was distributed exclusively in the nucleus, indicating that CCDC106 is a nuclear protein. Using the LOCtree software [21], we found that CCDC106 contains two putative NLS (NLS1, ^{151}RRR^{153} and NLS2, ^{162}RRR^{164}). To examine this prediction, we made a series of mutants and transfected them into HeLa cells. As shown in Fig. 2, deletion of the putative NLS (residues 151–164) resulted in cytoplasmic localisation of CCDC106 and exclusion of CCDC106 from the nucleus. Substitution of the arginines of both NLS1 and NLS2 with alanines also led to the exclusive cytoplasmic localisation of CCDC106, whereas site-directed mutagenesis of only one of both clusters of arginine residues led to a mixed distribution in both the cytoplasm and the nucleus, suggesting that the two clusters of basic residues in the NLS are indispensable for nuclear localisation of CCDC106. These results show that the region of residues 151–164 in the middle of CCDC106 is a bipartite NLS that is responsible for the nuclear localisation of CCDC106.

3.3. CCDC106 co-localises and interacts with p53 in the nucleus

CCDC106 was previously identified as a p53-binding partner by high-throughput automated yeast two-hybrid screening [18]. Therefore, we determined whether these two proteins share the same location in a cell using immunofluorescence staining. Consistent with the localisation of overexpressed EGFP-tagged CCDC106, endogenous CCDC106 protein is localised in the nucleus of HeLa cells (Fig. 3A). Interestingly, although both CCDC106 and p53 are predominantly localised in the nucleus, the protein signals are merged mainly in the mitotic (M) phase of the cell cycle (Fig. 3A), suggesting that the interaction of CCDC106 and p53 occurs mainly in M phase.

To further confirm the in vivo interaction between CCDC106 and p53, we performed co-immunoprecipitation experiments. Myc-tagged CCDC106 and HA-tagged p53 expression plasmids were introduced into HeLa cells. The cell lysates were precipitated with ether control IgG or anti-Myc polyclonal antibody, and the precipitated complex was detected for the presence of HA-p53 by western blotting using anti-HA monoclonal antibody. As shown in Fig. 3B, p53 could be detected in immune complexes precipitated by anti-Myc monoclonal antibody, but not by IgG, indicating that CCDC106 can interact with p53.

3.4. CCDC106 inhibits the transcriptional activity of p53

The tumour suppressor function of the p53 protein is mainly correlated with its ability to transcriptionally activate promoters containing p53-binding sites. Therefore, we studied the effects of CCDC106 overexpression on the transactivation activity of p53 in order to determine the functional significance of the binding of CCDC106 to p53. The pp53-TA-luc construct containing a p53 response element was transfected into HeLa cells, which express wild-type p53, together with increasing amounts of the CCDC106 expression plasmid pMyc-CCDC106. We found that transfection of CCDC106 significantly decreased the luciferase activity of pp53-TA-luc in a dose-dependent manner, suggesting CCDC106 inhibits the transactivation of p53 (Fig. 4).

3.5. CCDC106 stimulates p53 protein degradation

To elucidate the mechanism for the inhibited transcription activity of p53 by CCDC106, we then examined whether CCDC106 affected the level of p53 protein. HeLa cells were co-transfected with 2 μg of pHA-P53 and increasing amounts of pMyc-CCDC106 (0, 2, 4, 8 μg). The results show that with an increase of Myc-CCDC106 protein, the level of HA-p53 protein decreased rapidly, but no change in the level of endogenous β-actin protein was observed (Fig. 5A). These results indicate that CCDC106 decreases the p53 protein level. To further determine whether the reduced level of the p53 protein resulted from p53 protein degradation,

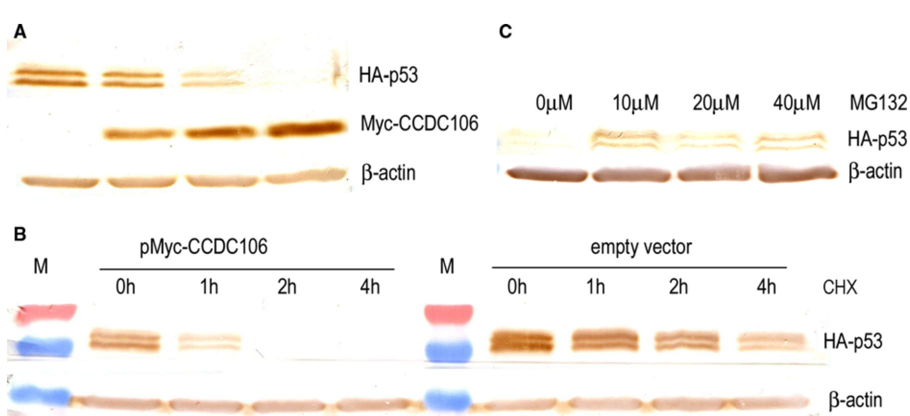

Fig. 5. CCDC106 promotes the proteasome-dependent degradation of p53 protein. (A) HeLa cells were transfected with 2 μg of pHA-p53 and increasing amounts of pMyc-CCDC106 (0, 2, 4, 8 μg) and harvested 8 h post-transfection. (B and C) HeLa cells were transfected with 2 μg of pHA-p53 and 4 μg of pMyc-CCDC106 or empty vector pCMV-Myc. At 8 h post-transfection, transfected cells were incubated with either CHX (50 μg/ml) for different lengths of time (0, 1, 2 and 4 h) (B), or CHX plus increasing concentrations of MG132 (0, 10, 20, 40 μM) for 1.5 h (C). Cell lysates were separated by 10% SDS–PAGE electrophoresis and transferred onto a PVDF membrane. The blot membrane was cut into strips and incubated with matched primary antibody as described in Section 2.

we compared the p53 protein degradation half-lives in HeLa cells transfected with pMyc-CCDC106 or empty vector pCMV-Myc. As shown in Fig. 5B, p53 was much more rapidly degraded in cells transfected with pMyc-CCDC106 than in cells with empty vector. However, treatment with the proteasomal inhibitor MG132 efficiently rescued the protein level of p53 (Fig. 5C), indicating that p53 is degraded in an ubiquitin-proteasome-dependent degradation manner. These results suggest that CCDC106 can increase the proteasome-dependent degradation of p53.

In conclusion, this study identified for the first time the putative gene CCDC106 as a functional gene through a series of experiments. We cloned three variant transcripts of the CCDC106 gene and identified the in vivo expression of its transcripts and protein in cancer cell lines. Furthermore, we discovered that CCDC106 is a novel negative regulator of p53. Loss of p53 function is a characteristic of almost all human tumours [3]. We found that CCDC106 is highly expressed in p53 wild-type cancer cell lines NCI-H292 and HeLa (Fig. 1C), suggesting that the ectopic expression of CCDC106 might contribute to tumourigenesis by blocking p53-mediated growth suppression and apoptosis. However, other p53 wild-type cancer cell lines such as HCT116 and MCF-7 express much low level of CCDC106. In these cell lines, p53 activity is possibly prevented by other cellular factors. For example, BCAS2 gene is upregulated by gene amplification in breast cancer cell lines MCF-7 [22,23]. BCAS2 protein directly interacts with p53 to reduce p53 transcriptional activity by mildly but consistently decreasing p53 protein [23]. Our studies concerning the activity of CCDC106 should be valuable toward understanding the process of tumourigenesis in cancers harbouring wild-type p53.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.02.031.

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